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(54) Title: METHOD FOR MOLECULAR CLONING AND POLYNUCLEOTIDE SYNTHESIS USING VACCINIA DNA TOPOISOMERASE (57) Abstract <p>This invention provides a modified vaccinia topoisomerase enzyme containing an affinity tag which is capable of facilitating purification of protein-DNA complexes away from unbound DNA. This invention further provides a modified sequence specific topoisomerase enzyme. This invention provides a method of ligating duplex DNAs, a method of molecular cloning of DNA, a method of synthesizing polynucleotides, and a method of gene targeting. Lastly, this invention provides a recombinant DNA molecule composed of segments of DNA which have been joined <i>ex vivo</i> by the use of a sequence specific topoisomerase and which has the capacity to transform a suitable host cell comprising a DNA sequence encoding polypeptide activity.</p>		

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METHOD FOR MOLECULAR CLONING AND POLYNUCLEOTIDE
SYNTHESIS USING VACCINIA DNA TOPOISOMERASE

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This application is a continuation of U.S. Serial No. 08/358,344 filed December 19, 1994, the contents of which are hereby incorporated by reference into the present application.

10

This invention was made with support under Grant No. GM-46330 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the United States Government has certain rights in the invention.

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Throughout this application, various publications are referenced by Arabic numerals in brackets. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are in their entirety hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

25

BACKGROUND OF THE INVENTION

Construction of chimaeric DNA molecules in vitro relies traditionally on two enzymatic steps catalyzed by separate protein components. Site-specific restriction endonucleases are used to generate linear DNAs with defined termini that can then be joined covalently at their ends via the action of DNA ligase.

30

Vaccinia DNA topoisomerase, a 314-aa virus-encoded eukaryotic type I topoisomerase [11], binds to duplex DNA and cleaves the phosphodiester backbone of one strand. The enzyme exhibits a high level of sequence specificity, akin to that of a restriction

35

endonuclease. Cleavage occurs at a consensus pentapyrimidine element 5'-(C/T)CCTT⁶ in the scissile strand [12, 5, 6]. In the cleavage reaction, bond energy is conserved via the formation of a covalent adduct between the 3' phosphate of the incised strand and a tyrosyl residue (Tyr-274) of the protein [10]. Vaccinia topoisomerase can religate the covalently held strand across the same bond originally cleaved (as occurs during DNA relaxation) or it can religate to a heterologous acceptor DNA and thereby create a recombinant molecule [7, 8].

The repertoire of DNA joining reactions catalyzed by vaccinia topoisomerase has been studied using synthetic duplex DNA substrates containing a single CCCTT cleavage site. When the substrate is configured such that the scissile bond is situated near (within 10 bp of) the 3' end of a DNA duplex, cleavage is accompanied by spontaneous dissociation of the downstream portion of the cleaved strand [4]. The resulting topoisomerase-DNA complex, containing a 5' single-stranded tail, can religate to an acceptor DNA if the acceptor molecule has a 5' OH tail complementary to that of the activated donor complex. Sticky-end ligation by vaccinia topoisomerase has been demonstrated using plasmid DNA acceptors with four base overhangs created by restriction endonuclease digestion [8].

SUMMARY OF THE INVENTION

- This invention provides
a modified vaccinia topoisomerase enzyme containing an
5 affinity tag which is capable of facilitating
purification of protein-DNA complexes away from unbound
DNA. This invention further provides a modified
sequence specific topoisomerase enzyme.
- 10 This invention provides a method of ligating duplex
DNAs, a method of molecular cloning of DNA, a method of
synthesizing polynucleotides, and a method of gene
targeting.
- 15 Lastly, this invention provides a recombinant DNA
molecule composed of segments of DNA which have been
joined ex vivo by the use of a sequence specific
topoisomerase and which has the capacity to transform
a suitable host cell comprising a DNA sequence encoding
20 polypeptide activity.

BRIEF DESCRIPTION OF THE FIGURES**Figures 1A-1C: Sticky-end ligation.**

5 Figure 1A: Topoisomerase-mediated cleavage of a
24-nucleotide CCCTT-containing hairpin substrate
was assayed as a function of enzyme concentration.
The structure of the substrate is shown; the site
of strand scission is indicated by the arrow.
10 Reaction mixtures (20 ml) containing 50 mM Tris
HCl (pH 7.5), 0.5 pmol of 5' ³²P-labeled DNA, and
topoisomerase were incubated at 37°C for 5 min.
Covalent complexes were trapped by addition of SDS
to 1%. Samples were then electrophoresed through
15 a 10% polyacrylamide gel containing 0.1% SDS.
Covalent complex formation was revealed by
transfer of radiolabeled DNA to the topoisomerase
polypeptide as detected by autoradiographic
exposure of the dried gel. The extent of adduct
20 formation was quantitated by scintillation
counting of an excised gel slice containing the
labeled protein and was expressed as the percent
of the input 5' ³²P-labeled oligonucleotide that
was covalently transferred to protein.

25 Figure 1B: Reaction mixtures containing 50 mM
Tris HCl (pH 7.5), 460 fmol of 5' ³²P-labeled
hairpin substrate, and 2 pmol of topoisomerase
were incubated for 5 min at 37°C, then
30 supplemented with linear pUC18 DNA acceptor (350
fmol of ends) as indicated and incubated for
another 5 min at room temperature. Samples were
adjusted to 0.2 M NaCl and 0.5% SDS, then
electrophoresed through a 1.2% agarose gel in TBE
35 (90 mM Tris, 90 mM borate, 2.5 mM EDTA) with 0.5
mg/ml ethidium bromide. DNA was visualized by

photographing the stained gel under short wave UV illumination.

5 Figure 1C: The same gel was then dried and
exposed for autoradiography. The positions of the
radiolabeled topoisomerase-DNA "donor" complex and
the pUC strand transfer product are indicated at
the right. pUC18 DNA used as acceptor in the
strand transfer reactions was linearized
10 quantitatively by digestion with a single-cut
restriction enzyme. The 5' phosphate termini of
the linear DNAs were converted to 5' OH ends by
treatment of the DNAs with calf intestinal
phosphatase as indicated (CIP). The acceptor DNAs
15 included in each reaction are specified according
to lane number. Lane M (left panel) contains DNA
size markers (1 HindIII digest).

20 Figure 2: Monovalent, bivalent, and trivalent
substrates.

The structure of the complementary hairpin
oligonucleotides S300 and S301 are shown. The 5'
25 terminus is indicated by an asterisk. The CCCTT
recognition site of topoisomerase cleavage is
underlined. The structure of the bivalent linker
DNA formed by annealing S300 and S301 strands is
shown in the middle. At bottom is the structure
30 of the trivalent Y-branched linker formed by
annealing S300, S304, and S303 oligonucleotides.

Figures 3A-3C: Topoisomerase-mediated cleavage of monovalent, bivalent, and trivalent substrates.

5 Figure 3A: Radiolabeled cleavage substrates were electrophoresed through a native polyacrylamide gel (7.5% acrylamide, 0.2% bisacrylamide) in TBE at 100 V. An autoradiogram of the dried gel is shown. Lane 1 contains the 5' ³²P-46-mer "flip" hairpin (S300; Fig. 2). Lane 2 contains the 46-bp
10 divalent cleavage substrate (Fig. 2). This structure was formed by annealing the 5' ³²P-S300 strand to a 3-fold molar excess of unlabeled 46-nt complementary strand (S301, or "flop" strand; Fig. 2). Lane 3 contains the trivalent Y-branch
15 substrate formed by annealing 5' ³²P-S300 to two unlabeled 46-mer oligos (S303 and S304), each present at 3-fold molar excess over the labeled strand.

20 Figure 3B: Cleavage reaction mixtures (20 ml) contained 50 mM Tris HCl (pH 7.5), 0.6 pmol of 5' ³²P-labeled DNA, and 20 pmol of topoisomerase (lanes 2, 4, 6, and 8) were incubated at 37°C for
25 5 min. Enzyme was omitted from control reactions (lanes 1, 3, 5, and 7). Covalent complexes were trapped by addition of SDS to 1%. (Note that the samples were not heat-denatured). Labeled cleavage products were resolved by SDS-PAGE. Free
30 DNA migrated with the bromophenol blue dye front. The structures of the various covalent protein-DNA complexes are indicated at the right of the autoradiogram. The positions and sizes (in kDa) of prestained marker proteins are indicated at the
35 left. The input substrates are illustrated at the bottom of the autoradiogram: *S300 (lanes 1 and

2); *S301 (lanes 3 and 4); *S300/S301 (lanes 5 and 6); S300/*S301 (lanes 7 and 8).

5 Figure 3C: Cleavage reactions contained 0.36 pmol of radiolabeled Y-branch substrate (*S300/S303/S304) and 20 pmol of topoisomerase (lane 2). Enzyme was omitted from a control reaction (lane 1). The structures of the various covalent protein-DNA complexes are indicated at the right of the autoradiogram. The positions and sizes (in kDa) of prestained marker proteins are indicated at the left.

15 Figures 4A-4B: Topoisomerase-mediated joining of two ends via a bivalent linker.

20 Figure 4A: Reaction mixtures (20 ml) contained 50 mM Tris HCl (pH 7.5), 2 pmol of topoisomerase, and either 5' ³²P-labeled monovalent substrate (*S300, 0.6 pmol - lanes 1 and 2) or 5' ³²P-labeled bivalent linker (0.3 pmol of *S300/S301, i.e., 0.6 pmol of ends - lanes 3 and 4). After incubation for 5 min at 37°C, the reactions were supplemented with 5'-OH HindIII-cut pUC18 DNA acceptor (380 fmol of ends) as indicated and incubated for another 5 min at room temperature. Samples were adjusted to 0.2 M NaCl and 0.5% SDS, then electrophoresed through a 1.2% agarose gel in TBE. The ethidium bromide stained gel is shown at left. 25 The positions and sizes (kbp) of marker DNA fragments (lane M) are indicated at the left.

30 Figure 4B: The same gel was dried and exposed for autoradiography. The positions of the radiolabeled topoisomerase-DNA "donor" complex and the strand transfer products are indicated at right by arrows.

Figures 5A-5D: Molecular cloning of DNA using vaccinia topoisomerase.

5 **Figure 5A:** Ligation reactions for topoisomerase-based cloning were performed as described under Experimental Details. The protocol is illustrated schematically.

10 **Figures 5B-5C:** Plasmid DNA was prepared from bacteria containing pUC18 (the parent vector, Fig. 5B) and pUC-T11 (a representative transformant from the topoisomerase ligation reaction, Fig. 5C). DNA was digested with the restriction endonucleases specified above each lane using
15 reaction buffers provided by the vendor. Undigested plasmid DNA is shown in Lane "---". Lane M contains DNA size markers. The positions and sizes (kbp) of reference fragments are indicated.

20 **Figure 5D:** The structure of the 46-bp bivalent linker is indicated. Diagnostic restriction sites within the linker are specified above the sequence.

25 **Figures 6A-6B:** Topoisomerase-mediated joining of two ends via a trivalent linker.

30 **Figure 6A:** Each strand of the trivalent substrate (Fig. 2) was 5' labeled and gel-purified. The Y-branched substrate was generated by annealing equimolar amounts of the three strands (*S300, *S303, *S304). The annealed product was analyzed by electrophoresis through a native 7.5% polyacrylamide gel. An autoradiograph of the gel
35 is shown. The trivalent substrate is in lane 3. Component strands were analyzed in parallel (*S303

in lane 1; *S304 in lane 2). The structures of the labeled species are indicated at the right.

5 Figure 6B: Reaction mixtures (20 ml) contained 50 mM Tris HCl (pH 7.5), 1 pmol of topoisomerase, and either 5' ³²P-labeled monovalent substrate (*S304 - lanes 1 and 2) or 5' ³²P-labeled trivalent linker (0.3 pmol of *S300/*S303/*S304 - lanes 3 and 4). Each reaction contained 350 fmol of input substrate (expressed as cleavable ends). After 10 incubation for 5 min at 37°C, the reactions were supplemented with 5'-OH HindIII-cut pUC18 DNA acceptor (570 fmol of ends) as indicated and incubated for another 5 min at room temperature. 15 Samples were adjusted to 0.2 M NaCl and 0.5% SDS, then electrophoresed through a 1.2% agarose gel in TBE. The ethidium bromide stained gel is shown. The positions and sizes (kbp) of marker DNA fragments (lane M) are indicated at the left.

20 Figure 6C: The same gel was dried and exposed for autoradiography. The positions of the radiolabeled topoisomerase-DNA "donor" complex and the strand transfer products are indicated at right by arrows 25 and brackets.

Figure 7: Expected products of bivalent end-joining.

30 The locations of restriction sites for HindIII (H), XmnI (X), SspI (S), and AccI (A) within the linear pUC acceptors and anticipated ligation products are indicated by arrows. The pUC DNA is denoted by a solid bar. The predicted sizes of SspI and XmnI restriction fragments derived from 35 each species are listed at the left. Fragments that are expected to contain radiolabeled linker DNA are indicated by asterisks.

Figure 8: Expected products of trivalent end-joining.

5 The expected products of trivalent end joining to pUC DNA are shown in the box. Digestion with XmnI is predicted to yield four trivalent products, which are depicted at the right. The lengths of the pUC "arms" (in kpb) are indicated.

10 **Figures 9A-9C: Restriction endonuclease digestion of end-joining reaction products.**

15 **Figure 9A:** Reaction mixtures (20 ml) contained 50 Mm Tris Hcl (pH 7.5), 1 pmol of topoisomerase, and either monovalent substrate (*S300 - lanes 1 and 2), divalent linker (*S300/*301 - lanes 3 and 4), or trivalent linker (*S300/*S303/*S304 - lanes 5 and 6). After incubation for 5 min at 37°C, the reactions were supplemented with either 5'-OH HindIII-cut pUC19 "bivalent" DNA acceptor (600 fmol linear DNA - lanes 1, 3, and 5) or 5'-OH HindIII/5'-P AccI-cut PUC19 "monovalent" acceptor (500 fmol of linear DNA - lanes 2, 4, and 6) and incubated for another 5 min at room temperature. The mixtures were adjusted to recommended restriction conditions by addition of 10x buffer concentrate (NEB2) and the samples were digested with SspI (10 units; New England BioLabs) for 60 min at 37°C. Samples were adjusted to 0.5% SDS and electrophoresed through a 1.2% agarose gel in TBE. An ethidium bromide stained gel is shown. The positions and sizes (kbp) of marker DNA fragments (lane M) are indicated at the left.

35 **Figures 9B-9C:** Cleavage reactions containing radiolabeled bivalent linker (lanes 1 and 2) or trivalent linker (lanes 3-5) were supplemented with divalent pUC19 acceptor (lanes 1 and 3) or

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5 monovalent pUC19 acceptor (lanes 2 and 4). A control reaction received no acceptor (lane 5). The strand transfer reaction products were digested with XmnI (40 units) for 2 h at 37°C, then analyzed by agarose gel electrophoresis. The ethidium bromide stained gel is shown (Fig. 9B). The positions and sizes (kbp) of marker DNA fragments (lane M) are indicated at the left of the photograph. The same gel was dried and exposed for autoradiography (Fig. 9C). The positions of the radiolabeled topoisomerase-DNA "donor" complex and the strand transfer products are indicated at right by arrows and brackets.

10

DETAILED DESCRIPTION OF THE INVENTION

5 This invention provides a modified vaccinia topoisomerase enzyme containing an affinity tag. The modified vaccinia topoisomerase enzyme is capable of facilitating purification of a vaccinia topoisomerase-DNA complex from unbound DNA. This invention also provides a modified sequence specific topoisomerase
10 enzyme. The sequence specific topoisomerase enzyme can be any site specific type I topoisomerase.

Topoisomerases are a class of enzymes that modify the topological state of DNA via the breakage and rejoining
15 of DNA strands. Vaccinia topoisomerase enzyme is a vaccinia virus-encoded eukaryotic type I topoisomerase. In one embodiment vaccinia topoisomerase enzyme is a 314 aa virus encoded type I topoisomerase.

20 In another embodiment the modified vaccinia enzyme is a site-specific type I topoisomerase. Site-specific type I topoisomerases include, but are not limited to, viral topoisomerases such as pox virus topoisomerases. Examples of pox virus topoisomerases include Shope
25 fibroma virus and ORF virus. Other site specific topoisomerases are known to those skilled in the art.

In another embodiment the affinity tag includes, but is not limited to, the following: a glutathione-S-
30 transferase fusion tag, a maltose binding protein tag, a histidine or poly-histidine tag.

In one embodiment the vaccinia topoisomerase-DNA complex is purified from unbound DNA by binding the
35 histidine tagged topoisomerase-DNA complex to a nickel column and eluting the substrate with imidazole.

This invention provides a duplex DNA molecule, that is, a double-stranded DNA molecule, having at each end thereof the modified vaccinia topoisomerase enzyme.

5 Vaccinia topoisomerase binds to duplex DNA and cleaves the phosphodiester backbone of one strand while exhibiting a high level of sequence specificity, cleaving at a consensus pentapyrimidine element 5'-(C/T)CCTT+, or related sequences, in the scissile
10 strand. In one embodiment the scissile bond is situated in the range of 2-12 bp from the 3' end of a duplex DNA. In another embodiment cleavable complex formation by vaccinia topoisomerase requires six duplex nucleotides upstream and two nucleotides downstream of
15 the cleavage site. Examples of vaccinia topoisomerase cleavable sequences include, but are not limited to, +6/-6 duplex GCCCTTATTCCC, +8/-4 duplex TCGCCCTTATTC, +10/-2 duplex TGTCGCCCTTAT, and +10/-2 duplex GTGTCGCCCTTA.

20 As used herein, the term donor signifies a duplex DNA which contains a CCCTT cleavage site within 10 bp of the 3' end and the term acceptor signifies a duplex DNA which contains a 5'-OH terminus. Once covalently
25 activated by topoisomerase the donor will only be transferred to those acceptor ends to which it can base pair.

This invention provides a method of ligating duplex
30 DNAs employing the modified tagged vaccinia topoisomerase. In this method of ligation the donor duplex DNA substrate is a bivalent donor duplex DNA substrate, that is, it contains two topoisomerase cleavage sites. One embodiment comprises cleaving a
35 donor duplex DNA substrate containing sequence specific topoisomerase cleavage sites by incubating the donor duplex DNA substrate with a sequence specific

topoisomerase to form a topoisomerase-bound donor duplex DNA strand and incubating the topoisomerase-bound donor duplex DNA strand with a 5' hydroxyl-terminated compatible acceptor DNA, resulting in the ligation of the topoisomerase-bound donor duplex DNA strand to the DNA acceptor strand.

Methods of cleaving DNA by incubation with enzymes and methods of ligating DNA by incubation are known to those skilled in the art. In one embodiment the sequence specific topoisomerase is a vaccinia topoisomerase enzyme. In another embodiment the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme. In embodiments using vaccinia or modified vaccinia topoisomerase enzyme the cleavage site is an oligopyrimidine motif 5' (C/T)CCTT↓.

In one embodiment the desired subpopulation of DNA ligation product is purified by introducing to the 5' end of the donor duplex DNA an affinity label. In a preferred embodiment the affinity label is a biotin moiety and purification is performed by binding the biotin-ligated product to streptavidin. Other purification methods are known to those skilled in the art.

Bivalent end-joining allows the assembly of linear concatamers from polynucleotides with compatible ends. When the linker is designed to generate the same overhang at each cleavage site, the strand transfer products are randomly oriented as head-to-head, head-to-tail, and tail-to-tail isomers. Control of the reaction can be easily achieved by using a bivalent linker containing different overhangs at each cleavage site; in this way, DNA acceptors prepared with two different restriction enzymes can be assembled in a strictly head-to-tail fashion. The ligation can be

made exclusively head-to-head by combining a symmetric bivalent linker with an acceptor DNA containing asymmetric ends.

5 Bivalent strand transfer also results in circularization of the acceptor, a property that can be exploited for molecular cloning. For example, by placing the topoisomerase cleavage sites on the insert (a synthetic bivalent substrate) and cloning the
10 cleaved DNA into a plasmid vector. This strategy is well-suited to the cloning of DNA fragments amplified by PCR. To clone PCR products using vaccinia topoisomerase, it is necessary to include a 10-nucleotide sequence -5'-XXXXAAGGGC- at the 5' end of
15 the two primers used for amplification. The 5'-XXXX segment can correspond to any 4-base overhang that is compatible with the restriction site into which the PCR product will ultimately be cloned. The amplification procedure will generate duplex molecules containing the
20 sequence -GCCCTT^axxxx-3' at both 3' ends (where xxxx is the complement of XXXX). Incubation of the PCR product with topoisomerase will result in cleavage at both termini and allow the covalently activated PCR fragment to be ligated to vector DNA, essentially as described
25 in Figure 5A.

This invention also provides a method of molecular cloning of DNA. One embodiment comprises introducing to a donor duplex DNA substrate a sequence specific
30 topoisomerase cleavage site by PCR amplifying the donor duplex DNA molecule with oligonucleotide primers containing the sequence specific topoisomerase cleavage site; incubating the donor duplex DNA with a sequence specific topoisomerase, resulting in the formation of
35 a sequence specific topoisomerase-donor duplex DNA complex; incubating the sequence specific topoisomerase-donor duplex DNA complex with a plasmid

vector with a 5' overhang compatible to the donor;
incubating the sequence specific topoisomerase-donor
duplex DNA complex with the plasmid vector; and
transforming the plasmid vector that has been incubated
5 into a host cell.

In one embodiment the sequence specific topoisomerase
is a vaccinia topoisomerase enzyme. In another
embodiment the sequence specific topoisomerase is a
10 modified vaccinia topoisomerase enzyme. In embodiments
using vaccinia or modified vaccinia topoisomerase
enzyme the cleavage site is an oligopyrimidine motif 5'
(C/T)CCTT↓.

15 PCR amplification methods are known to those skilled in
the art. In one embodiment, the cloning of PCR
products using vaccinia topoisomerase requires
including a 10-nucleotide sequence 5'-XXXXAAGGGC- at
the 5' end of the two primers used for amplification.
20 The 5'-XXXX segment can correspond to any 4-base
overhang compatible with the restriction site into
which the PCR product will be cloned. The
amplification procedure will generate duplex molecules
containing the sequence -GCCCTT*xxx-3' at both 3' ends
25 (where xxx is the complement of XXXX). Incubation of
the PCR product with topoisomerase results in cleavage
at both termini and allows the covalently activated PCR
fragment to be ligated to vector DNA.

30 Regulatory elements required for expression include
promoter or enhancer sequences to bind RNA polymerase
and transcription initiation sequences for ribosome
binding. For example, a bacterial expression vector
includes, but is not limited to, a promoter such as the
35 lac promoter and for transcription initiation the
Shine-Dalgarno sequence and the start codon AUG.
Similarly, a eukaryotic expression vector includes, but

- is not limited to, a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such
- 5 vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general.
- 10 In this invention transformation of the plasmid vector is into a prokaryotic host cell, such as a bacteria cell. In a preferred embodiment the host cell is E. coli.
- 15 Topoisomerase-based cloning has several advantages over conventional ligase-based cloning of PCR products. First, the topoisomerase procedure circumvents any problems associated with addition of nontemplated
- 20 nucleotides by DNA polymerase at the 3' end of the amplified DNA. Any nontemplated base (N) at the 3' end of a PCR product destined for topoisomerase-based cloning (GCCCTT^{3'}~~xxxx~~N-3') will dissociate spontaneously upon covalent adduct formation, and will therefore have no impact on the ligation to vector. Second, in
- 25 topoisomerase-mediated cloning, the only molecule that can possibly be ligated is the covalently activated insert and the insert can only be transferred to the vector. There is no potential for in vitro covalent closure of the vector itself, which ensures low
- 30 background. There is also no opportunity for the inserts to ligate to each other (this can be guaranteed by using 5'-phosphate-terminated PCR primers), which precludes cloning of concatameric repeats. Third, there is no need to consider the sequence of the DNA
- 35 being amplified in designing the PCR primers. It is commonplace in standard cloning to introduce a restriction site into the PCR primer and to cleave the

PCR products with that restriction enzyme to facilitate joining by ligase to vector. In cases where the sequence between the primers is not already known, it becomes problematic to choose a site for the primer that is not present in the amplified segment. This issue becomes even more relevant as PCR methodology advances and very long targets (10-40 kbp) are amplified routinely. The issue of internal topoisomerase cleavage sites (CCCTT or related pentapyrimidine elements) is not a significant impediment to topoisomerase-based cloning. This is because the cleavage-religation equilibrium at internal sites strongly favors the noncovalently bound state, and at those sites that are incised, only one strand of the duplex is nicked. Internal cleavage sites can be induced to religate by raising the salt concentration, which serves to dissociate noncovalently bound topoisomerase and drive the reaction equilibrium to the left. In contrast, cleavage at sites near the 3' end is virtually quantitative and is essentially irreversible until an acceptor DNA is provided.

Topoisomerase-based cloning strategies need not be limited to covalent activation of the insert. By designing a plasmid polylinker such that CCCTT sites are situated in inverted orientation on either side of a restriction site, one can generate a linear vector with topoisomerase sites at both 3' ends. Once covalently activated by topoisomerase, the vector "donor" can be used to clone any complementary insert "acceptor" (which must have 5'-OH termini), thereby precluding religation of the vector without the insert. It is worth noting that the donor complex formed upon cleavage by topoisomerase at a 3' proximal site is extremely stable. The donor molecule can be transferred nearly quantitatively to a complementary acceptor even after many hours of incubation of the

covalent topo-DNA complex at room temperature. Indeed, the topo-linker complex can be denatured with 6 M guanidine HCl and then renatured spontaneously upon removal of guanidine with complete recovery of strand transferase activity. Thus, a topoisomerase-activated vector can be prepared once in quantity and used as many times as needed for molecular cloning.

This invention provides a method of synthesizing polynucleotides. One embodiment comprises annealing a multiple number of duplex DNA strands to form a branched substrate containing a sequence specific topoisomerase cleavage site at each 3' end; cleaving the branched substrate by incubation with a sequence specific topoisomerase to form a branched topoisomerase complex; and incubating the branched topoisomerase complex with complementary monovalent and/or bivalent DNA acceptors. This method of polynucleotide synthesis is useful for in vitro end-labelling, ligand tagging, molecular cloning.

In one embodiment the sequence specific topoisomerase is a vaccinia topoisomerase enzyme. In another embodiment the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme. In embodiments using vaccinia or modified vaccinia topoisomerase enzyme the cleavage site is an oligopyrimidine motif 5' (C/T)CCTT↓.

In one embodiment annealing of the duplex DNA strands is performed by mixing the DNA strands and heating to 65°C for 5 minutes, and then allowing the mixture to slow cool to room temperature. One skilled in the art knows the procedures to follow for annealing duplex DNA.

In one embodiment three duplex DNA strands are used which form a trivalent Y-branched structure. Production of a Y-branched nucleic acid by the strand transfer reaction containing the trivalent linker can be demonstrated by diagnostic restriction digestion of the reaction products. The yield of Y-branched products can be optimized by eliminating residual bivalent and monovalent linkers from the substrate preparation or by ensuring that all trivalent linkers were saturated with three bound topoisomerase molecules. Both conditions can be met, by gel-purifying the linker and by purifying the tri-covalently activated species by sedimentation. As with bivalent ligation, the orientation of the Y-branched products can be controlled by manipulating the design of the linker, or by using asymmetric acceptors. Any head-to-head-to-head type Y-branched product of trivalent strand transfer can, in theory, be organized into a trivalent lattice by adding a second trivalent donor complex that is complementary to the "tail" of the original acceptor DNA. Donor substrates of higher order valence can be used to achieve topo-based synthesis of three dimensional lattices and polyhedra from DNA. Topoisomerase-based synthesis offers a potentially powerful alternative strategy for building complex biopolymers.

In one embodiment a duplex DNA strand is 5' labeled and the 5' labeled duplex DNA strand is annealed to the two duplex DNA strands to enable radiochemical purification of the substrate. Methods of radiochemical purification are known to those skilled in the art.

This invention provides a method of gene targeting. Gene targeting involves the introduction of DNA into a cell. The DNA is taken up into the chromosomal DNA by virtue of a topoisomerase-bound donor duplex DNA. The

bound topoisomerase seals the donor DNA to chromosomal DNA. One embodiment comprises cleaving a bivalent donor duplex DNA substrate containing a sequence specific topoisomerase cleavage site by incubating the donor duplex DNA substrate with a sequence specific topoisomerase to form a topoisomerase-bound donor duplex DNA strand; and transfecting the topoisomerase-bound donor duplex DNA to a suitable cell.

10 In one embodiment the sequence specific topoisomerase is a vaccinia topoisomerase enzyme. In another embodiment the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme. In embodiments using vaccinia or modified vaccinia topoisomerase enzyme the cleavage site is an oligopyrimidine motif 5' (C/T)CCTT+.

Transfection may be performed by any of the standard methods known to one skilled in the art, including, but not limited to electroporation, calcium phosphate transfection or lipofection.

This invention provides a recombinant DNA molecule composed of segments of DNA which have been joined ex vivo or in vitro by the use of a sequence specific topoisomerase and which has the capacity to transform a suitable host cell comprising a DNA sequence encoding polypeptide activity.

30 In one embodiment the sequence specific topoisomerase is a vaccinia topoisomerase enzyme. In another embodiment the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme. In embodiments using vaccinia or modified vaccinia topoisomerase enzyme the cleavage site is an oligopyrimidine motif 5' (C/T)CCTT+.

This invention is further illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS:

I. Methods

5

A) Enzyme Purification:

Vaccinia DNA topoisomerase was expressed in Escherichia coli and purified as described [9]. The heparin agarose enzyme fraction used in the present study was the same preparation described previously [9]. The enzyme was nearly homogeneous with respect to the 33 kDa topoisomerase polypeptide, as determined by SDS-polyacrylamide gel electrophoresis. Protein concentration was determined using the Biorad dye reagent, taking bovine serum albumin as the standard.

15

B) Synthesis of 5' Labeled Oligonucleotide Substrates:

20

Synthesis of DNA oligonucleotides via DMT-cyanoethyl phosphoramidite chemistry was performed by the Sloan-Kettering Microchemistry Laboratory using an Applied Biosystems model 380B or model 394 automated DNA synthesizer according to protocols specified by the manufacturer. Oligonucleotides containing the CCTT cleavage motif were labeled at the 5' end via enzymatic phosphorylation in the presence of [32 P]ATP and T4 polynucleotide kinase. Reaction mixtures (25 ml) typically contained 50 mM Tris HCl (pH 8.0), 10 mM dithiothreitol, 10 mM MgCl₂, 0.1 mM ATP, 100 mCi [32 P]ATP, T4 polynucleotide kinase (20 units, Bethesda Research Laboratories), and 500 pmol of DNA oligonucleotide (DNA was quantitated by A₂₆₀). Incubation was for 60 min at 37°C. Labeled DNA was freed of protein and radioactive nucleotide by electrophoresis through a non-denaturing 18%

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polyacrylamide gel. Full-sized labeled oligonucleotide was localized by autoradiographic exposure of the wet gel and the labeled DNA was recovered from an excised gel slice by soaking the slice in 0.4 ml H₂O for 8 h at room temperature. Hybridization of labeled DNAs to complementary oligonucleotides was performed in 0.2 M NaCl by heating to 75°C followed by slow cooling to room temperature. Annealed substrates were stored at 4°C.

C) Topoisomerase-based Cloning:

Reaction mixtures containing 50 mM Tris HCl (pH 7.5), 2 pmol of topoisomerase, and either monovalent linker (0.6 pmol) or bivalent linker (0.3 pmol) were incubated for 5 min at 37°C. A control reaction contained topoisomerase but no DNA substrate. Each mixture was then supplemented with 5'-OH HindIII-cut pUC18 DNA acceptor (380 fmol of ends) and incubated for another 5 min at room temperature. An aliquot (1 ml) of each sample was used to transform *E. coli* DH5a using a BioRad Gene Pulser electroporation apparatus. Preparation of bacterial cells and electrotransformation were carried out as prescribed by the manufacturer. Aliquots of transformed bacteria were plated on LB agar containing 0.1 mg/ml ampicillin.

II. Example 1. Sticky end Ligation:

The vaccinia topoisomerase was capable of sticky-end ligation of duplex DNAs containing only 2 bases of potential complementarity, as shown in Fig. 1. In this experiment, the "donor" was a 24-mer hairpin oligonucleotide containing a single CCCTT motif (a "monovalent" substrate) with the scissile bond located 2 bases from the 3' blunt end (Fig. 1A). The extent of cleavage of this substrate was proportional to enzyme

concentration (Fig. 1A). The topoisomerase-DNA complex migrated as a discrete species during native agarose gel electrophoresis (Fig. 1C). Addition of unlabeled 5' hydroxyl-terminated CpG tailed linear pUC18 DNA (generated by digestion of pUC DNA with AccI followed by treatment with alkaline phosphatase) resulted in transfer of the topoisomerase-bound DNA strand to the linear DNA "acceptor." The product of the strand transfer reaction was a radiolabeled 2.7 kbp linear form containing a hairpin end (Fig. 1C, lane 2). AccI-restricted plasmid DNA containing a 5'-phosphate terminus was inert as an acceptor (Fig. 1C, lane 3). [The requirement for a 5'OH-terminated acceptor excluded the possibility that the reaction products might be formed by a conventional DNA ligase contaminating the topoisomerase preparation]. Linear plasmid DNA containing non-complementary 5'-OH overhangs generated by restriction with EcoRI (5'-AATT) or HindIII (5'-AGCT) were ineffective as acceptors (Fig. 1C, lanes 4 and 6), as was 5'-OH blunt-ended linear DNA generated by restriction with SmaI (lane 5).

III. Example 2. Divalent Linkers as Donors:

Two 46-mer DNA strands were annealed to form a "divalent" 46-bp substrate containing a topoisomerase cleavage site 4 nucleotides from each 3' end (Fig. 2). Successful annealing of the constituent strands was evinced by the reduced mobility of the duplex molecule during native gel electrophoresis (Fig. 3A, lane 2) compared to that of the hairpin DNA (Fig. 3A, lane 1). Either the "flip" or "flop" monovalent hairpins were readily cleaved by vaccinia topoisomerase, resulting in the formation of a covalent protein-DNA adduct which migrated at 43 kDa during SDS-PAGE (Fig. 3B, lanes 2 and 4). Incubation of topoisomerase with the divalent duplex substrate yielded two complexes of 46 kDa and 72

kDa; the 46 kDa species represents a single molecule of topoisomerase bound covalently at one of the CCCTT cleavage sites; the 72 kDa complex arises by cleavage at both sites on the same DNA molecule (Fig. 3B, lanes 5 6 and 8).

The monovalent hairpin DNA was transferred virtually quantitatively to linear pUC DNA containing a complementary 5'-OH-AGCT overhang (Figs. 4A-4B, lane 10 2). Incubation of the bivalent topoisomerase-DNA complex with the same acceptor yielded a complex set of products arising from ligation of the bivalent linker to two complementary ends of the linear pUC acceptor (Figs. 4A-4B, lane 4). These included circular pUC and 15 linear pUC concatamers. A significant fraction of the pUC acceptor molecules were subject to bivalent end-joining, as reflected in the distribution of EtBr-stained DNA products (Fig. 4A, lane 4). All ligation events were via the radiolabeled linker DNA, which 20 became incorporated into the reaction products (Fig. 4B, lane 4).

IV. Example 3. Molecular Cloning of DNA Using Vaccinia Topoisomerase:

25 The ability of topoisomerase to join both ends of a linear DNA to a complementary acceptor suggested an alternative approach to molecular cloning. In the scheme shown in Fig. 5, the "insert" was a bivalent 46- 30 bp linker containing CCCTT sites at both 3' ends. The sequence of the linker included restriction sites for endonucleases NdeI, BglII, and EcoRV. Cleavage of the bivalent linker by topoisomerase generated a 4-base overhang complementary to a HindIII restriction site. 35 The "vector" was pUC DNA that had been cleaved with HindIII and dephosphorylated with alkaline phosphatase. Addition of the vector to the bivalent topoisomerase-

DNA donor complex should result in covalent joining of the insert to the vector. Upon transformation into E. coli, those molecules that had been circularized should be able to give rise to ampicillin-resistant colonies.

5 It was found that the yield of ampicillin-resistant colonies from bacteria transformed with a topoisomerase reaction mixture containing linear pUC and the bivalent linker was 110-fold higher than that observed for bacteria transformed with control topoisomerase

10 reactions containing linear pUC and either monovalent linker or no linker.

Plasmid DNA was recovered from cultures of six individual transformants and analyzed by restriction

15 endonuclease digestion in parallel with pUC18 plasmid DNA (Fig. 5B). [The restriction pattern for the recombinant clone pUC-T11 shown in Fig. 5C was indistinguishable from that of the five other clones, which are not shown]. Whereas the starting pUC18

20 plasmid contains no sites for EcoRV and BglII, the recombinant clone contains a single site for each enzyme, attributable to the insertion of the bivalent linker, which contains these restriction sites. Similarly, the starting plasmid contains a single NdeI

25 site, whereas the recombinant clone contains a second NdeI site in the linker insert. The size of the novel NdeI fragment in pUC-T11 indicated that the linker DNA was inserted within the pUC polylinker as expected. This was confirmed by the finding that the recombinant

30 plasmid had lost the original HindIII site upon strand transfer by topoisomerase to the HindIII overhang (the strand transfer reaction should generate the sequences AAGCTA and TAGCTT at the plasmid-insert junctions, which would not be cut by HindIII). The restriction

35 site for SphI, which is located immediately next to the HindIII site in the polylinker, was retained in all recombinant clones (not shown), indicating that loss of

the HindIII site was not caused by deletions occurring during strand transfer. Thus, the bivalent linker DNA was successfully cloned into the pUC18 vector in a simple procedure that - exclusive of the bacterial transformation step - takes only 10 minutes to execute.

V. Example 4. Trivalent Linkers as Donors:

Three 46-mer DNA strands were annealed to form a "trivalent" Y-branched substrate containing a topoisomerase cleavage site 4 nucleotides from each 3' end (Fig. 2). To optimize radiochemical purity of the substrate, one of the strands was 5' radiolabeled and annealed to the two other strands, which were present in molar excess (Fig. 3A). The radiolabeled Y-branched substrate migrated more slowly than a 46-bp linear duplex molecule during native gel electrophoresis (Fig. 3A, lane 3). Anomalous electrophoretic behavior of the Y molecule was also evident during SDS-PAGE, where the trivalent substrate migrated at a position equivalent to a 39 kDa protein (Fig. 3C, lane 1). The Y-branch structure was cleaved quantitatively upon incubation with topoisomerase; three complexes were resolved, corresponding to Y-molecules with one, two, or three covalent bound topo polypeptides (Fig. 3C). Most of the cleaved DNAs contained two or three bound topoisomerase molecules.

To test strand transfer by the trivalent donor complex, the Y-branched molecule was prepared by annealing equimolar amounts of the constituent strands, each of which was radiolabeled. Although the three-strand Y-form constituted the predominant product of the annealing reaction (Fig. 6A, lane 3), bivalent linkers were present as well (these molecules contain an unpaired "bubble" as indicated in Fig. 6). The radiolabeled substrate was transferred quantitatively

from the topoisomerase-DNA donor complex to a linear pUC18 acceptor containing a complementary 5'-OH-AGCT overhang (Fig. 6C, compare lanes 3 and 4). A complex array of multivalent ligation products was apparent by EtBr-staining and by autoradiography (Figs. 6B-6C, lane 4). These included circular pUC and linear pUC concatamers as well as higher order structures (the species indicated by the bracket in Fig. 6C). None of the concatamers or higher order forms were observed in a control strand transfer reaction containing a monovalent DNA linker (Figs. 6B-6C, lane 2).

VI. Example 5. Characterization of the Trivalent Strand Transfer Products:

The recombinant molecules generated by topoisomerase-mediated end-joining were analyzed further by digestion with restriction endonucleases that cleave once within the pUC sequence. In Fig. 7, the anticipated products of bivalent end-joining by topoisomerase are shown, along with the restriction fragments expected for each product upon digestion with SspI and XmnI. The products of trivalent end-joining are illustrated in Fig. 8. Experimental results showing the spectrum of strand transfer products after digestion with SspI and XmnI are shown in Fig. 9. In this analysis, each linker, which upon cleavage generated a tailed donor complex compatible with a HindIII restriction site, was tested with two acceptor molecules, one bivalent and one monovalent. The bivalent acceptor was linear pUC19 containing 5'-OH HindIII overhangs on both ends. Strand transfer of a polyvalent linker to the bivalent acceptor allows for the formation of circular and linear concatamers in a head-to-head, tail-to-tail, or head-to tail fashion, as shown in Fig. 7. The monovalent acceptor was pUC19 containing a 5'-OH HindIII site at one end and a 5'-phosphate AccI site at

the other end. Transfer of the linker by topoisomerase to the AccI terminus is precluded completely on two grounds; first, because the ends are not complementary and second, because topoisomerase cannot religate to a 5'-phosphate strand. A monovalent acceptor will react with the topoisomerase donor complex at available compatible termini, but will not be able to form circles or concatameric arrays. The structures of the various species can thus be inferred by direct comparison of the restriction digests from reaction in which monovalent, bivalent, and trivalent linkers were reacted with monovalent and bivalent acceptors.

Consider the SspI digests of topoisomerase strand transfer products in Fig. 9A. The monovalent linker was joined to either end of the bivalent pUC19 acceptor, but could not support circularization or dimerization. Hence the products were cleaved by SspI to yield two fragments derived from linear monomers (Fig. 9A, lane 1) (see Fig. 7). Ligation of the bivalent linker to bivalent acceptor yielded three additional products, a 4.1 kbp fragment diagnostic of head-to-head multimer formation, a 1.3 kbp fragment indicative of tail-to-tail ligation, and a 2.7 kbp species that derived from a circular molecule (Fig. 9A, lane 3). Ligation of the bivalent linker to a monovalent acceptor yielded the 4.1 kbp head-to-head fragment, but no fragments indicative of tail-to-tail or circular products (Fig. 9A, compare lanes 3 and 4). This was precisely as expected, because the AccI "tail" was inert for strand transfer. Reactions containing the trivalent Y-linker and bivalent acceptor yielded two novel high molecular weight products not observed for the bivalent linker (Fig. 9A, lane 5). The largest product (indicated by the arrowhead in Fig. 9A), which was also observed with trivalent linker and monovalent acceptor (Fig. 9A, lane 6), must correspond to a Y-

branched recombinant containing three pUC molecules ligated in head-to head fashion. The length of each arm is predicted to be 2 kbp. The electrophoretic mobility of this species was anomalously slow, as
5 expected for a branched DNA. The higher order complex unique to the bivalent acceptor was presumed to be a Y-branched product containing pUC19 DNA ligated in a mixed head-head and head-tail orientation.

10 Digestion of the strand transfer products with XmnI confirmed and extended these findings (Figs. 9B-9C). The digest of a reaction containing labeled bivalent linker and unlabeled bivalent pUC acceptor yielded
15 diagnostic linear fragments of 3.7 kbp (head-to-head multimer), 1.7 kbp (tail-to-tail multimer) and 2.7 kbp (circle). These products were detected by EtBr-staining and by autoradiography (Fig. 9B, lanes 1). The 1.7 kbp species indicative of tail-to-tail ligation
20 migrated just ahead of a 1.85 fragment. (derived either from end-tagged linear monomers or from head-to-tail multimers). The 1.7 kbp species was absent from the digest of products formed with the monovalent pUC acceptor (Fig. 9B, lanes 2). Similarly, the 2.7 kbp
25 species and the radiolabeled 0.8 kbp fragment (diagnostic of ligation to the "tail" end of pUC) were absent from the monovalent acceptor digest (Fig. 9B, lane 2).

30 The XmnI digest of products formed with labeled trivalent linker and bivalent pUC19 acceptor contained four unique species not seen with the bivalent linker (Fig. 9B, compare lanes 3 and 1). Three of these
35 molecules were readily apparent as high molecular weight EtBr-stained bands. The fourth species migrated barely in advance of the head-to-head linear fragment and was best appreciated in the autoradiograph (Fig. 9C, lane 3). These molecules correspond to the four

possible Y-branch structures shown in Fig. 8. A priori, if there was no bias in ligation orientation, one would expect a 1:3:3:1 distribution of head-head-head, head-head-tail, head-tail-tail, and tail-tail-tail isomers. Indeed, this is what was observed experimentally (Fig. 9B, lane 3). Consistent with the predicted structures of the Y-branched products, only the largest species (head-head-head) was detected in the reaction of trivalent linker with monovalent pUC acceptor.

REFERENCES:

- 5 1. Chen, J., and Seeman, N. C. (1991) Nature 350: 631-633.
2. Cheng, S., et al. (1994) Proc. Natl. Acad. Sci. USA 91: 5695-5699.
- 10 3. Clark, J. M. (1988) Nucleic Acids Res. 16: 9677-9686.
4. Morham, S. G., and Shuman, S. (1992) J. Biol. Chem. 267: 15984-15992.
- 15 5. Shuman, S. (1991a) J. Biol. Chem. 266: 1796-1803.
6. Shuman, S. (1991b) J. Biol. Chem. 266: 11372-11379.
- 20 7. Shuman, S. (1992a) J. Biol. Chem. 267: 8620-8627.
8. Shuman, S. (1992b) J. Biol. Chem. 267: 16755-16758.
- 25 9. Shuman, S., et al. (1988) J. Biol. Chem. 263: 16401-16407.
- 30 10. Shuman, S., et al. (1989) Proc. Natl. Acad. Sci. USA 86: 9793-9797.
11. Shuman, S., and Moss, B. (1987) Proc. Natl. Acad. Sci. USA 84: 7478-7482.
- 35 12. Shuman, S., and Prescott, J. (1990) J. Biol. Chem. 265: 17826-17836.
- 40 13. Stivers, J. T., et al. (1994) Biochemistry 33: 327-339.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Shuman, Stewart
- (ii) TITLE OF INVENTION: METHOD FOR MOLECULAR CLONING AND
POLYNUCLEOTIDE SYNTHESIS USING VACCINIA DNA
TOPOISOMERASE
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
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 - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
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- (viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCCCTTATTC CC

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGCCCTTAT TC

12

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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12

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGTCGCCCT TA

12

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

-36-

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

NNNNAAGGGC

10

What is claimed is:

- 5 1. A modified vaccinia topoisomerase enzyme
 containing an affinity tag.
- 10 2. The composition of claim 1, wherein the
 modified vaccinia topoisomerase enzyme is
 capable of facilitating purification of a
 modified vaccinia topoisomerase-DNA complex
 from unbound DNA.
- 15 3. The composition of claim 1, wherein the
 affinity tag is a histidine tag.
4. A duplex DNA molecule having at each end
 thereof a modified topoisomerase enzyme of
 claim 1.
- 20 5. The modified vaccinia topoisomerase enzyme
 of claim 1, wherein the modified vaccinia
 topoisomerase enzyme is a sequence specific
 topoisomerase.
- 25 6. A method of ligating duplex DNAs
 comprising:
 - 30 a) cleaving a bivalent donor duplex
 DNA substrate containing a
 sequence specific topoisomerase
 cleavage site, by incubating the
 donor duplex DNA substrate with a
 sequence specific topoisomerase
 to form a topoisomerase-bound
35 donor duplex DNA strand; and

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- 5 b) incubating the topoisomerase-bound donor duplex DNA strand with a 5' hydroxyl-terminated compatible acceptor DNA, resulting in the ligation of the topoisomerase-bound donor duplex DNA strand and the DNA acceptor strand.
- 10 7. The method of claim 6, wherein the sequence specific topoisomerase is a vaccinia topoisomerase enzyme.
- 15 8. The method of claim 6, wherein the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme.
- 20 9. The method of claim 6, wherein the sequence is a CCCTT cleavage site.
10. The method of claim 6, wherein the CCCTT cleavage site is within about 10 bp of the 3' end of the DNA duplex.
- 25 11. The method of claim 6, further comprising, purifying the desired subpopulation of DNA molecules by introducing a biotin moiety
- 30 12. The method of claim 11, further comprising, binding the ligated product to streptavidin.
13. The method of claim 6, wherein the 5' end of the donor duplex DNA contains an affinity label.
- 35 14. The method of claim 13, wherein the affinity label is a biotin moiety.

15. A method of molecular cloning DNA comprising:

- 5 a) introducing to a donor duplex DNA substrate a sequence specific topoisomerase cleavage site by PCR amplifying the donor duplex DNA molecule with oligonucleotide primers containing the cleavage site;
- 10 b) incubating the donor duplex DNA with a sequence specific topoisomerase, resulting in the formation of a sequence specific topoisomerase-donor duplex DNA complex;
- 15 c) incubating the sequence specific topoisomerase-donor duplex DNA complex with a plasmid vector with a 5' overhang compatible to the donor;
- 20 d) incubating the sequence specific topoisomerase-donor duplex DNA complex with the plasmid vector; and
- 25 e) transforming the plasmid vector of step (c) into a host cell.
- 30

35 16. The method of claim 15, further comprising the donor duplex DNA of step (a) wherein the donor duplex DNA contains sequence specific topoisomerase cleavage sites at both 3' ends.

- 5 17. The method of claim 15, further comprising the plasmid of step (c), wherein the plasmid vector contains the sequence specific topoisomerase cleavage sites situated in inverted orientation on either side of a restriction enzyme cleavage site.
- 10 18. The method of claim 15, wherein the sequence specific topoisomerase is a vaccinia topoisomerase enzyme.
- 15 19. The method of claim 15, wherein the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme.
- 20 20. The method of claim 15, wherein the sequence is a CCCTT cleavage site.
21. The method of claim 15, wherein the CCCTT cleavage site is within about 10 bp of the 3' end of the DNA duplex.
- 25 22. A method of synthesizing polynucleotides comprising:
- 30 a) annealing a multiple number of duplex DNA strands to form a branched substrate containing a sequence specific topoisomerase cleavage site at each 3' end;
- 35 b) cleaving the branched substrate by incubation with a sequence specific topoisomerase to form a branched topoisomerase complex; and

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c) incubating the branched topoisomerase complex with complementary monovalent and/or bivalent DNA acceptors.

5

23. The method of claim 22, wherein three duplex DNA strands are used.

10

24. The method of claim 23, wherein the three duplex DNA strands form a trivalent Y-branched structure.

15

25. The method of claim 24, further comprising, eliminating residual bivalent and/or monovalent linkers from the Y-branched substrate by gel purifying the substrate.

20

26. The method of claim 25, further comprising, purifying the polynucleotide of step (b) by sedimentation.

25

27. The method of claim 22, wherein the sequence specific topoisomerase is a vaccinia topoisomerase enzyme.

30

28. The method of claim 22, wherein the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme.

29. The method of claim 22, wherein the sequence is a CCCTT cleavage site.

35

30. The method of claim 22, wherein the CCCTT cleavage site is within about 10 bp of the 3' end of the DNA duplex.

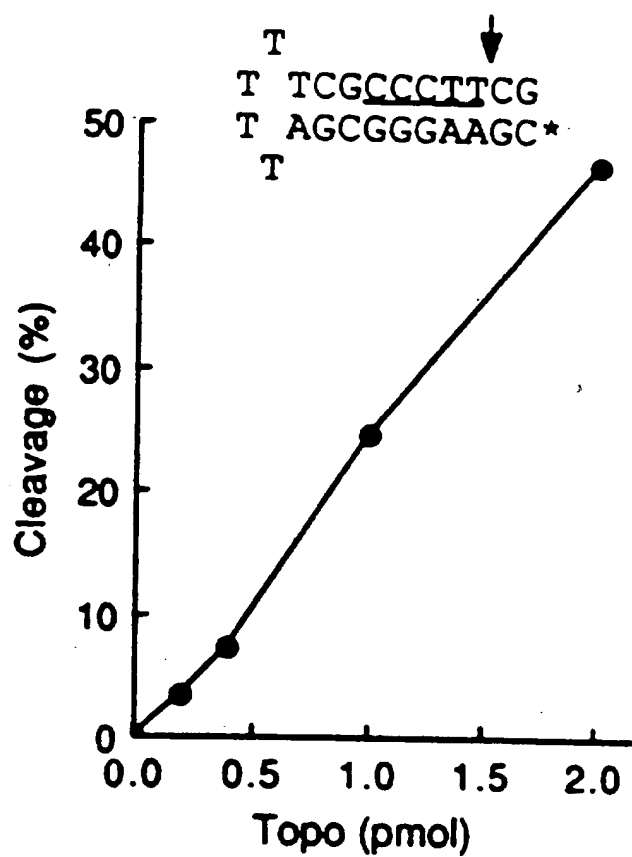
31. The method of claim 24, wherein a duplex DNA strand is 5' labeled.
- 5 32. The method of claim 31, further comprising annealing the 5' labeled duplex DNA strand to the two duplex DNA strands in order to radiochemically purify the substrate.
- 10 33. A method of gene targeting which comprises:
- a) cleaving a bivalent donor duplex DNA substrate containing a sequence specific topoisomerase cleavage site, by incubating the donor duplex DNA substrate with a sequence specific topoisomerase to form a topoisomerase-bound donor duplex DNA strand; and
- 15 b) transfecting the topoisomerase-bound donor duplex DNA to a suitable cell.
- 20 34. The method of claim 32, wherein the sequence specific topoisomerase is a vaccinia topoisomerase enzyme.
- 25 35. The method of claim 33, wherein the sequence specific topoisomerase is a modified vaccinia topoisomerase enzyme.
- 30 36. The method of claim 33, wherein the sequence is a CCCTT cleavage site.
- 35 37. The method of claim 33, wherein the CCCTT cleavage site is within about 10 bp of the 3' end of the DNA duplex.

5

38. A recombinant DNA molecule composed of segments of DNA which have been joined ex vivo or in vitro by the use of a sequence specific topoisomerase and which has the capacity to transform a suitable host cell comprising a DNA sequence encoding polypeptide activity.

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FIGURE 1A



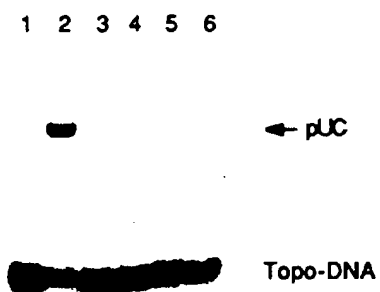
2/12

FIGURE 1B



1. No acceptor
2. pUC-AccI/CIP
3. pUC-AccI

FIGURE 1C



4. pUC-Eco/CIP
5. pUC-Sma/CIP
6. pUC-Hind/CIP

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FIGURE 2

<p>S300</p> <p>TTCGATATCATC</p> <p>A GCCCTTAGCT</p> <p>A CGGGAATCGA*</p> <p>GTCTAGAGTATA</p>	<p>S301</p> <p>CAGATCTCATAT</p> <p>T GCCCTTAGCT</p> <p>T CGGGAATCGA*</p> <p>AAGCTATAGTAG</p>
--	--

<p>*AGCTAAGGGCATATGAGATCTGAATTCGATATCATCGCCCTTAGCT</p> <p>TCGATTCCCGTATACTCTAGACTTAAGCTATAGTAGCGGGAATCGA*</p>	<p>S300</p> <p>S301</p>
---	-------------------------

<p>AGCTAAGGGCATATGAGATCTGAATTCGATATCATCGCCCTTAGCT</p> <p>TCGATTCCCGTATACTCTAGACTTAAGCTATAGTAGCGGGAATCGA</p>	<p>S300</p>
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<p>S304</p>	<p>TA</p> <p>TA</p> <p>GC</p> <p>GC</p> <p>TA</p> <p>AT</p> <p>CG</p> <p>CG</p> <p>AT</p> <p>TA</p> <p>GC</p> <p>GC</p> <p>TA</p> <p>CG</p> <p>GC</p> <p>GC</p> <p>GC</p> <p>AT</p> <p>AT</p> <p>TA</p> <p>CG</p> <p>GC</p> <p>AT</p>	<p>S303</p>
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FIGURE 3C

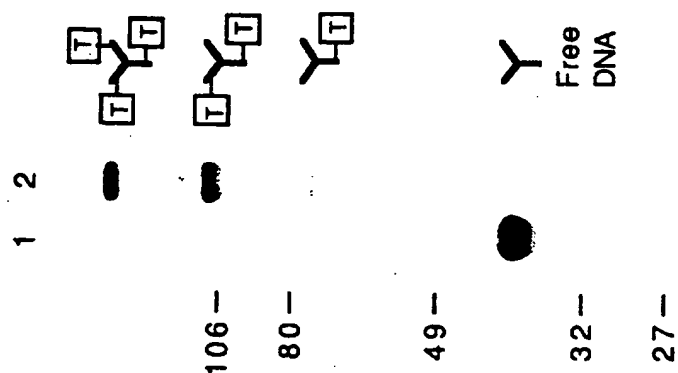


FIGURE 3B

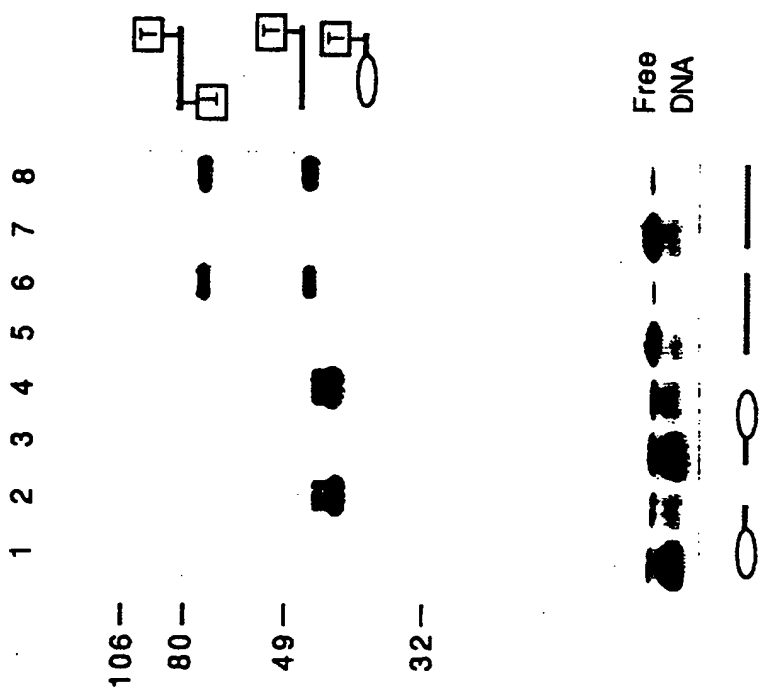
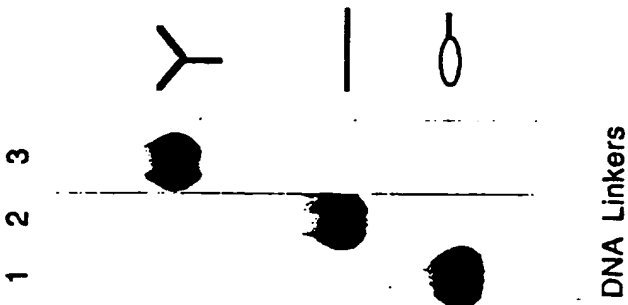
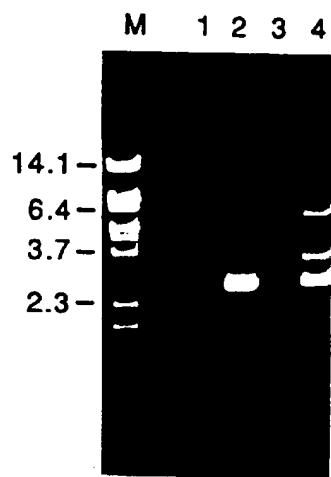


FIGURE 3A



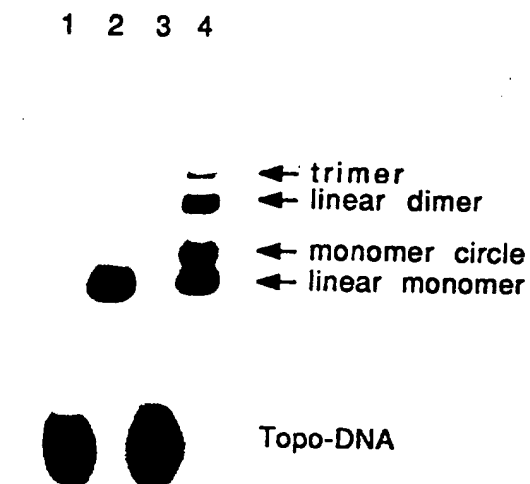
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FIGURE 4A



1. No acceptor
2. pUC-Hind/CIP

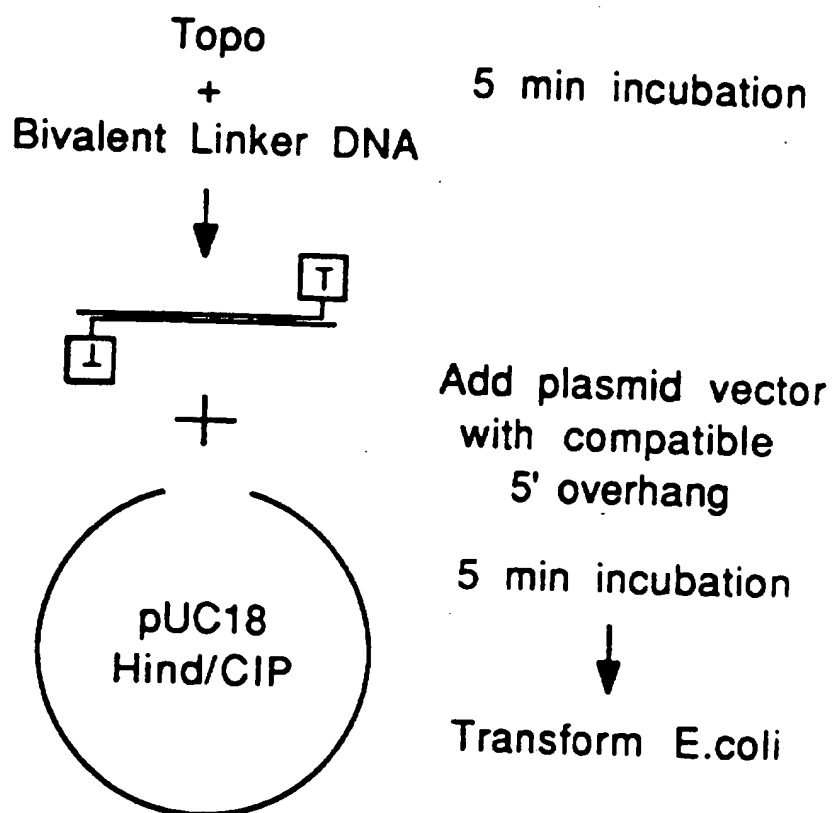
FIGURE 4B



3. No acceptor
4. pUC-Hind/CIP

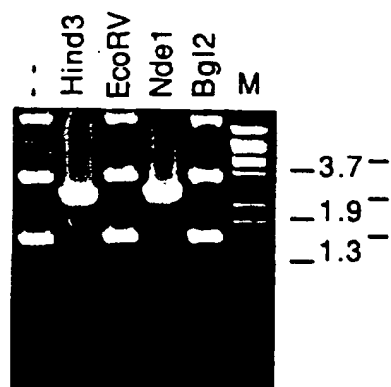
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FIGURE 5A



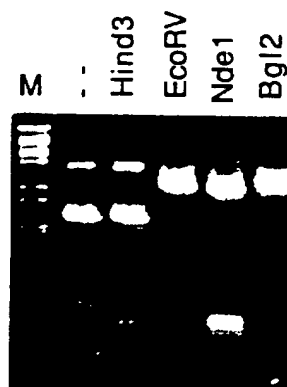
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FIGURE 5B



pUC18

FIGURE 5C



pUC-T11

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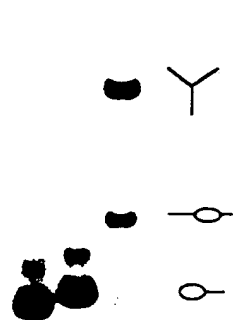
FIGURE 5D

NdeI *BglII* *EcoRV*
AGCTAAGGGCATATGAGATCTGAATTCGATATCATCGCCCTTAGCT
TCGATTCCCGTATACTCTAGACTTAAGCTATAGTAGCGGGAATCGA

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FIGURE 6A

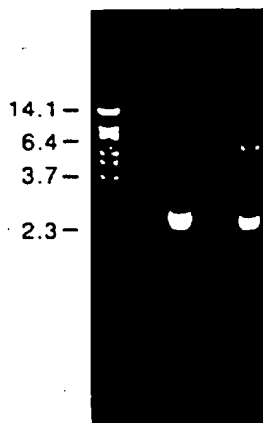
1 2 3



DNA Linkers

FIGURE 6B

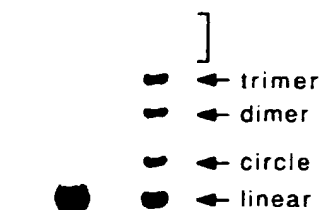
M 1 2 3 4



- 1. No acceptor
- 2. pUC-Hind/CIP
- 3. No acceptor
- 4. pUC-Hind/CIP

FIGURE 6C

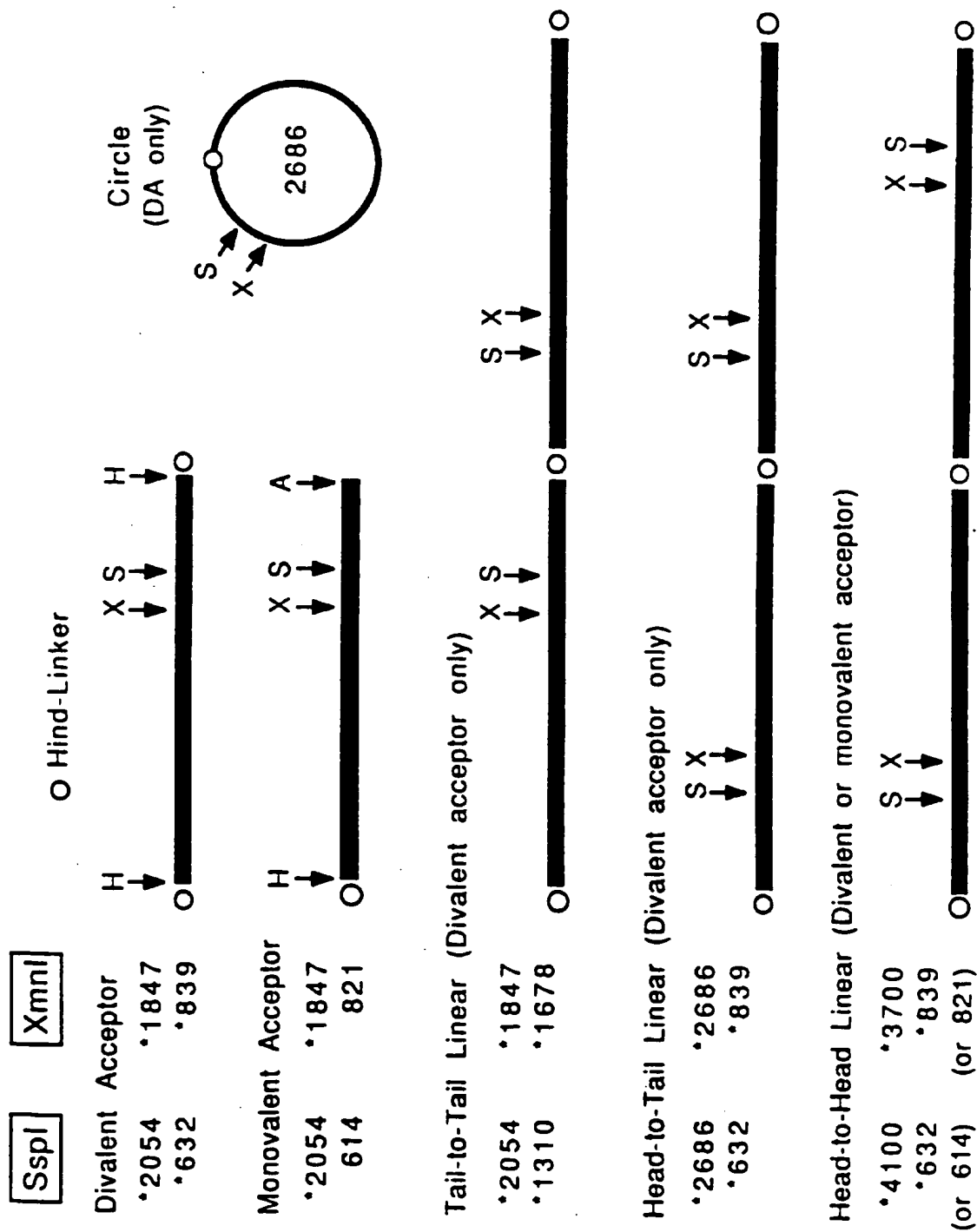
1 2 3 4



Topo-DNA

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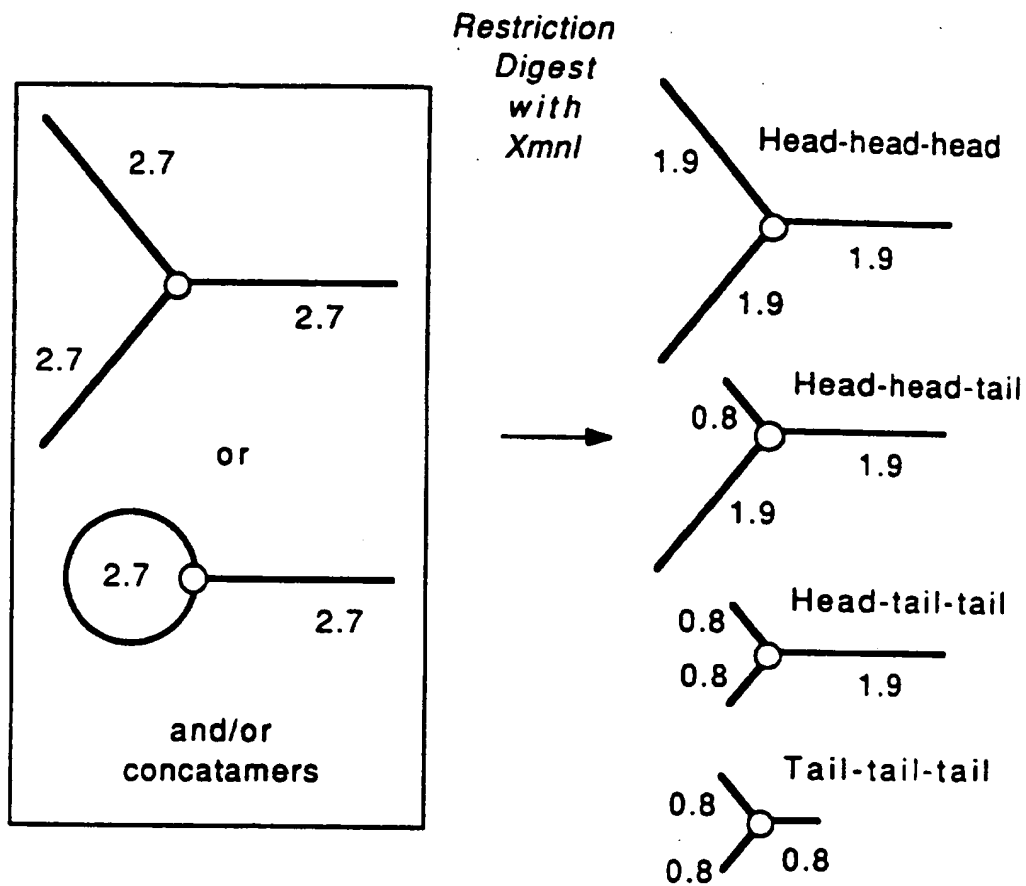
FIGURE 7



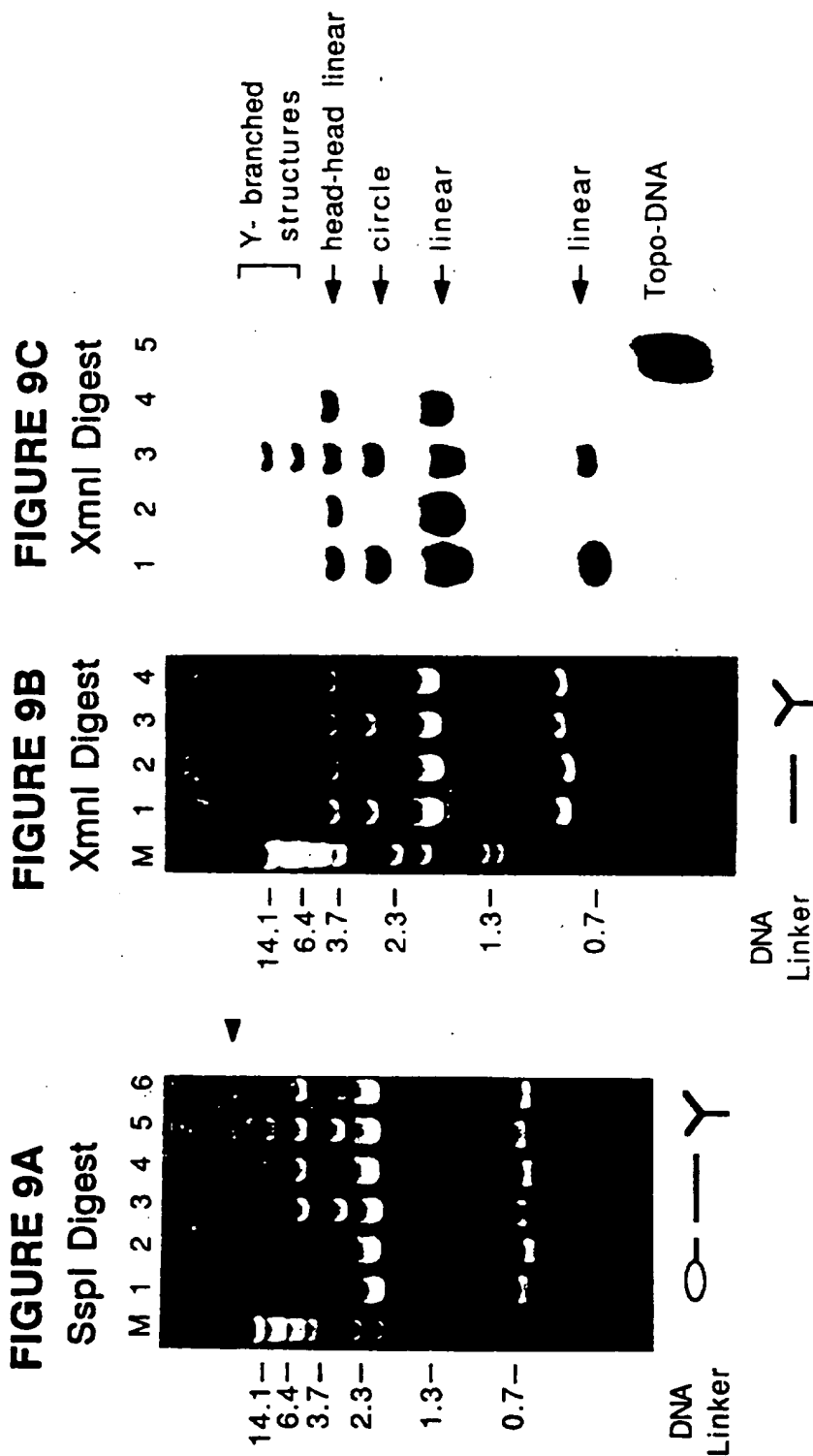
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FIGURE 8

Y-branched Ligation Products with Trivalent Linker



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/16099

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/07; C12N 15/09, 15/66; C12P 19/34; C07H 21/04

US CL : 435/91.41, 91.5, 172.3; 530/350; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.41, 91.5, 172.3; 530/350; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, USA, Vol. 84, issued November 1987, Shuman et al, "Identification of a vaccinia virus gene encoding a type I DNA topoisomerase", pages 7478-7482, see page 7481.	1-32, 38
X --- Y	The Journal of Biological Chemistry, Vol. 267, No. 24, issued 25 August 1992, Shuman, "Two Classes of DNA End-joining Reactions Catalyzed by Vaccinia Topoisomerase I", pages 16755-16758, see entire document, especially page 16758.	6, 7, 9, 10 ----- 8, 11-21, 38

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

03 APRIL 1996

Date of mailing of the international search report

24 APR 1996

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, A ----- Y, A	Biochemistry, Vol. 33, issued 1994, Stivers et al, "Vaccinia DNA Topoisomerase I: Single-Turnover and Steady-State Kinetic Analysis of the DNA Strand Cleavage and Ligation Reactions", pages 327-339, see entire document.	6, 7, 9, 10 ----- 4, 8, 11-14
A	The Journal of Biological Chemistry, Vol. 266, No. 17, issued 15 June 1991, Shuman, "Site-specific Interaction of Vaccinia Virus Topoisomerase I with Duplex DNA", pages 11372-11379.	6-38
X, P ----- Y, P	The Journal of Biological Chemistry, Vol. 269, No. 51, issued 23 December 1994, Shuman, "Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase", pages 32678-32684, see entire document especially page 32678.	6, 7, 9-18, 20-27, 29-32, 38 ----- 4, 8, 19, 28
Y	Molecular Biology Reports, Vol. 18, No. 3, issued October 1993, Schmitt et al., "Affinity purification of histidine-tagged proteins", pages 223-230, see pages 223, 228 and 229.	1-5, 8, 19
Y	Proceedings of the National Academy of Science USA, Vol. 88, issued October 1991, Janknecht et al., "Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus", pages 8972-8976, see pages 8972-8973.	1-5, 8, 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16099

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, EMBASE, MEDLINE, CAPLUS

search terms: topoisomerase#, Shuman, S, affinity tag?, histidine, glutathione s transferase, maltose binding, fusion, cloning, ligat?, DNA join?, (sequence or site) (w) specific?

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